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Depression

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Effects of long-term stress and recovery on the prefrontal cortex and dentate gyrus in male and female rats

(Submitted)

Abstract Women show a higher prevalence for depression than men, and stress plays an important role in the development of depression. However, the biological basis of the gender differences and the effects of recovery after long-term stress still remain poorly understood. Therefore, the aim of the present study was to assess the gender difference in response to acute stress (1 day), chronic stress (42 days) and recovery (stressed for 21 days, then recovered for another 21 days) using behavioral, endocrine and neurochemical parameters. Our results showed that stress decreased the male body weight while had no effect on female rats. Open field test demonstrated that chronic stress increased the grooming frequency both in male rats and female rats. Chronic and acute stress also increased the activity of HPA axis reflected by adrenal hypertrophy and increase of corticosterone levels except in the socially-housed female rats. Gender and brain region differences in response to stress and recovery were found in the expression of cAMP response element-binding protein (CREB) and phosphorylated CREB (pCREB). On the whole, expression of CREB and pCREB in male dentate gyrus (DG) and prefrontal cortex (PFC) was sensitive while in female DG and PFC it was resistant to acute and chronic stress. Interestingly, recovery restored the measured parameters to the normal level in male rats. However, in female rats we did not find such recovery after 3-week's. In conclusion, these results suggest that male and female rats responded to stress and recovery in a different way.

Keywords stress, gender difference, neuroplasticity, social housing, dentate gyrus, prefrontal cortex

Introduction

Depression, a common public health problem, occurs twice as frequently in women as in men (Kessler et al, 1993; Kessler, 2003; Sun and Alkon 2006). Such a gender difference in depression may occur for a number of reasons, including the influence of particular sex hormones (Matheson and Anisman, 2003). In rodents, females typically respond to a stress with a greater release of both adrenal corticotropic hormone (ACTH) and corticosterone compared to males (Rivier et al, 1999; Tinnikov et al, 1999). Dysregulation of the hypothalamic-pituitary- adrenocortical (HPA) axis is associated with vulnerability to a number of psychiatric diseases including major depression (Howell and Muglia, 2006). There are also anatomical differences and fMRI studies show different patterns of brain activity in males and females. Therefore, the gender differences in stress reactions and depression derived from both clinical and preclinical studies are an important argument for performing stress and pharmacological studies not only in male animals but also in female animals (Renard et al, 2005). The cellular, molecular and the psychosocial mechanisms underlying stress responses and depression may differ between males and females (Sjoberg et al, 2006). However, many experimental studies focusing on the pathophysiology of depression have examined the effects of stress and/or antidepressants in male subjects (Palanza, 2001), and the gender differences in the pathophysiology of depression remain poorly understood.

The onset of major depression is often preceded by chronic stress or stressful life events, indicating the importance of stress in the development of depression (Bale, 2006). However, the cellular and molecular mechanisms underlying depression have been difficult to grasp due to the complex pathophysiology of depression. In recent years, the focus of research has been at a level beyond the serotonin and norepinephrine transporters to the intracellular signal transduction cascades that underlie the regulation of neuronal functioning (Tardito et al, 2006). Novel theories suggest that dysregulation of intracellular pathways is linked to neuroplasticity as key events in depression development (Duman et al, 1997; Manji et al, 2001). One of the action mechanisms of stress may involve the dysregulation of the cyclic adenosine monophosphate (cAMP) cascade (Duman et al, 1997). This upregulation extends to several components of the cascade: coupling of stimulatory G-protein and adenylate cyclase (Ozawa and Rasenick, 1991), cAMP-dependent protein kinase (Nestler et al, 1989), transcription factor cAMP response element binding protein (CREB) (Nibuya et al, 1996) and brain derived neurotrophic factor (BDNF) (Nibuya et al, 1995). Among them CREB is a critical mediator of neural plasticity and has been implicated in learning, memory and the long-term actions of opiates, psychostimulants, as well as antidepressants (Newton et al, 2002). Phosphorylation of CREB (pCREB) at serine 133 has been reported to lead to activation of gene transcription, such as BDNF (Carlezon et al, 2005), therefore, nuclear

staining of pCREB reports activation of a particular brain area in response to external or internal stimuli, as does nuclear staining immediate early genes (IEG), like fos. Despite the importance of CREB and pCREB involved in neuroplasticity, few studies were designed to elucidate the gender differences in CREB and pCREB expression induced by stress and recovery after long-term stress.

Chronic footshock exposure has been proposed as a valid animal model for affective disorders (Westenbroek et al, 2003b). Chronic stress impairs the ability to anticipate reward which resembles anhedonia and can be reversed by antidepressants (Willner, 1997). At the same time, evidence show that some patients who respond to antidepressants also respond to placebo and there is natural recovery after depression, (Vallance, 2007). However, gender differences in recovery after long-term footshock exposure remain unknown.

To address these issues above, we attempted to determine the neurobiological adaptations underlying acute stress, long-term stress and recovery effects on individually- housed male and female rats. At the same time, behaviorally, females seem to have adopted a "tend- befriend" strategy, actively seeking social contact in times of stress (Taylor et al, 2000), so we also used socially-housed female rats to control for isolation stress in female rats. Particular focuses were on the prefrontal cortex (PFC) and dentate gyrus (DG), giving that studies in humans and animals have demonstrated that depression and stress-induced pathologies are implicated in DG and PFC, the key regions for HPA axis glucocorticoid feedback (Popoli et al, 2002; Sheline 2003; Radley et al, 2004; Sairanen et al, 2007). These relationships were studied by using markers of HPA axis function, behavioral test, and expression of CREB and pCREB. The present results may thus provide novel insights into cellular and molecular mechanisms underlying effects of stress, recovery on neuronal functions in the male and female rats.

Materials and Methods

Animals

Male (n=32) and female (n=48) Wistar rats were used in the present experiment. At the start of the experiment, rats were of the same age with male rats weighing 204±1.5 g and female rats weighing 186±1 g. All male rats and 32 female rats were individually housed, 16 female rats were socially housed (4 rats/cage), with ad libitum access to food and water. A plastic tube (diameter 8×17 cm.) was placed in each cage as a shelter. The light-dark cycle was reversed (lights on 19:00 - 7:00 h). All experimental procedures, designed to minimize the number of animals and suffering, were approved by the Animals Ethics Committee of the University of Groningen (FDC: D4445). The estrus cycle of the female rats was monitored by stroking them gently on the back,

producing lordosis during estrus and weight loss was observed on the day of estrus. In the current study effects of the estrous cycle on the stress response were not specifically investigated. It was hypothesized that since female rats were exposed to the stressor during all stages of the cycle this would overrule sex hormone related stress sensitivity differences (Westenbroek et al, 2003b).

Individually-housed male and female rats were randomly assigned to four experimental groups: 1; Control group: subjected to no footshock throughout the experiment, 2; Acute stress group: received 6 footshocks on day 42, and exposed to the footshock box with the light stimulus only on day 43, 3; Recovery group: received footshocks daily for three weeks followed by a 3-week period with no footshock, and on day 43 exposure to the footshock box with only the light and no shock, 4; Chronic stress group: received footshocks daily for three weeks followed by 3 weeks of alternating days of exposure to the footshock box with footshocks and without receiving footshock, and on day 43 exposure to the footshock box with only the light (**Fig.1**). Because we have previously shown that isolation was a stressor for female rats, we had 2 extra socially housed groups of female rats: a social control group that was not exposed to the footshock box throughout the experiment, and a social acute stress group, in which rats received 6 footshocks on day 42 and were exposed only to the light on day 43. This was to determine whether isolation for 6 weeks resulted in a changed stress response.

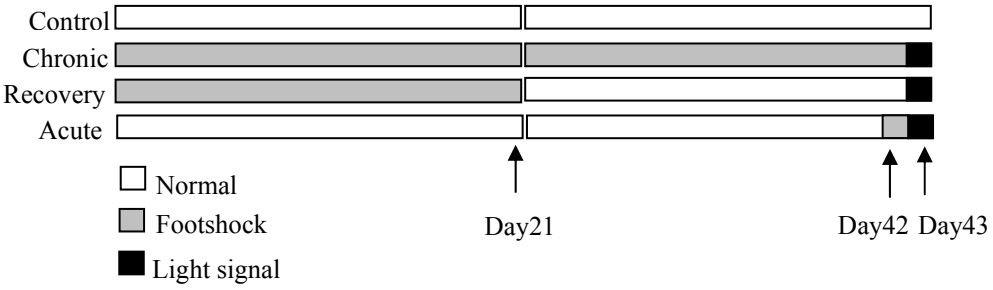


Figure 1. Schematic overview of the 43-day protocol. Control: rats were subjected to no footshock; Acute: rats received 6 footshocks on day 42, and were exposed to the footshock box with the light stimulus only on day 43; Recovery: rats received footshocks daily for three weeks followed by a 3-week period with no footshock, and on day 43 were exposed to the footshock box with the light only; Chronic: rats received footshocks daily for three weeks followed by 3 weeks of alternating days of exposure to the footshock box with footshocks and without receiving footshock, and on day 43 were exposed to the footshock box with the light only.

Stress procedure

The “footshock chamber” consists of a box containing an animal space positioned on a metallic grid floor connected to a shock generator and scrambler. Rats in the stress group were placed in a box and received variable (2-6) inescapable footshocks with randomized starting time (between 9:00 a.m. and 5:00 p.m.) and intervals during a 30-120 min session (0.8 mA as maximum intensity and 8 s in duration) in order to make the procedure as unpredictable as possible. A light signal (10 s) preceded each footshock adding a ‘psychological’ component to the stressor. On the last day, the stress-exposed animals were subjected to the light stimulus only, which was crucial as it provided a way to create a stress condition without the unwanted side effects of direct physical or painful stimuli. Body weights were measured daily during the whole period. Blood samples were collected through the tail vein quickly on day 20 and stored at -20°C to determine plasma corticosterone levels.

On day 43 rats were sacrificed using isoflurane anesthesia. Three rats from each group were transcardially perfused with 50 ml heparinized saline and 300 ml of a 4% paraformaldehyde solution in 0.1 M sodium phosphate buffer (pH 7.4), 2 hours after the start of the last exposure to the stress box, blood samples were taken by cardiac puncture. The brains were postfixed in the same fixative overnight at 4°C. The other five rats from each group were decapitated 30 minutes after the start of the last exposure to the stress box and the brains were removed immediately and put on dry ice and stored at -80°C, blood samples were also collected. The blood samples were centrifuged and plasma was removed and stored at −20°C. Adrenals and thymus were removed. Adrenal and thymus weights, corrected for body weight, were calculated and used as indication of the amount of stress perceived.

Open field test

Animals were subjected to a voluntary open field test (OF) for a period of 8 min. The open field test was performed under red-light conditions between 9 a.m. - 1 p.m. during the active period of the animals, at least 16 h after the last stress session and before the stress procedure of that day. The test was repeated 3 times, on days 1, 22, and 40. Rats were gently placed in the tube which was connected with the open field at the start of the test. The open field consisted of a circular black arena with a diameter of 1.5 m. The behavior of rats was recorded via a videotracking system (EthoVision 3.0®, Noldus information Technology, Wageningen, the Netherlands), with a sample rate of 25 samples/s. Grooming frequency in the whole arena was analyzed.

Corticosterone radioimmunoassay

Blood samples were collected for corticosterone radioimmunoassay (RIA). The corticosterone fraction was extracted from the plasma (10µl) with Chromosorb (Alttech) and 30% dichloromethane (Rathburn 1001). Before the extraction a trace amount of ^{3}H labeled (200 Bq) corticosterone (TRK-406 Amersham) was added to the samples to determine recovery which was 20%. A standard curve was determined by using non-labeled corticosterone, which was treated in the same way as the extracted plasma samples. For the quantification samples were mixed with ^{3}H (500 Bq) labeled corticosterone and polyclonal antibody raised against corticosterone (rabbit nr 568 UMCG). The tubes were incubated at 60°C for 30 minutes and the equilibrium reaction was established in a water/ice bath for 1 hour. The reaction was stopped by adding charcoal suspension and incubated for 15 min in the water/ice bath. The tubes were centrifuged for 15 minutes at 3000 g and 4°C to spin down the charcoal bound with free corticosterone. The supernatant was poured into 1 ml of scintillation fluid (Ultimagold XR, Canberra Packard) and samples were counted in a β-counter for 4 minutes or 4000 preset counts. The amount of corticosterone was calculated from the standard curve.

Western blot analysis

Serial 300 µm coronal sections of the cerebrum were made with a cryostat microtome (-15°C) and kept frozen on dry ice. Tissue samples were dissected from the DG and PFC (DG: bregma -2.45 to -2.85; **Fig. 2**) by using the "Palkovits Punch" technique (needle diameter 1.22 mm, Stoelting Co., Illinois, USA). Two punches per animal per area were taken and homogenized in 50 µl buffer (50 mM Tris pH 6.8, 1% SDS, 5% β-mercaptoethanol, 1 mM $NaVO_4$, 10mM NaF, 1mM EGTA and complete protease inhibitors Roche). Brain tissue was homogenized and then heated for 2 min at 96°C. The samples were stored at -80°C.

The expression of CREB, pCREB and β-actin as a loading control was estimated by Western blot analysis. Protein samples (5µl) were combined with SDS- bromophenol blue (5µl) reducing buffer with 100 mM DTT and then heated for 5 min at 96°C to limit the formation of high molecular weight receptor aggregates. Western blot analyses were carried out using 10% SDS polyacrylamide gels; gels were electroblotted (Supported Nitrocellulose Membrane, Biorad) for 1 h using a wet electroblotting system (Biorad, Mini Trans-Blot Electrophoretic Transfer Cell), and filters were blocked for 1 h in PBS buffer (pH 7.4) containing 0.5% non-fat dry milk. Blots were then incubated overnight at 4 °C with primary polyclonal antibodies of CREB, pCREB (Upstate Biotechnology, Lake Placid, NY) and β-actin (Abcam, Cambridge, UK) (1:1000 for CREB and pCREB, 1:10000 for β-actin) diluted in 0.5% non-fat dry milk-PBS solution. Blots were washed

three times for 10 min with PBS buffer and then incubated for 1 h with secondary antibodies (peroxidase-coupled anti-rabbit for CREB and pCREB and peroxidase-coupled anti-mouse for β-actin, Amersham Bioscience UK) diluted (1:5000) with 0.5% non-fat dry milk-PBS. Immunostaining was revealed by the enhanced ECL Western Blot analysis system (Syngene, Westburg, The Netherlands). The intensity of the bands was quantitated by image analysis. Membranes were washed and re-probed with the antibody to *β*-actin for loading control. The CREB and pCREB signals were normalized by the levels of β-actin and expressed as a percentage of the corresponding values for unstressed controls on the same blots.

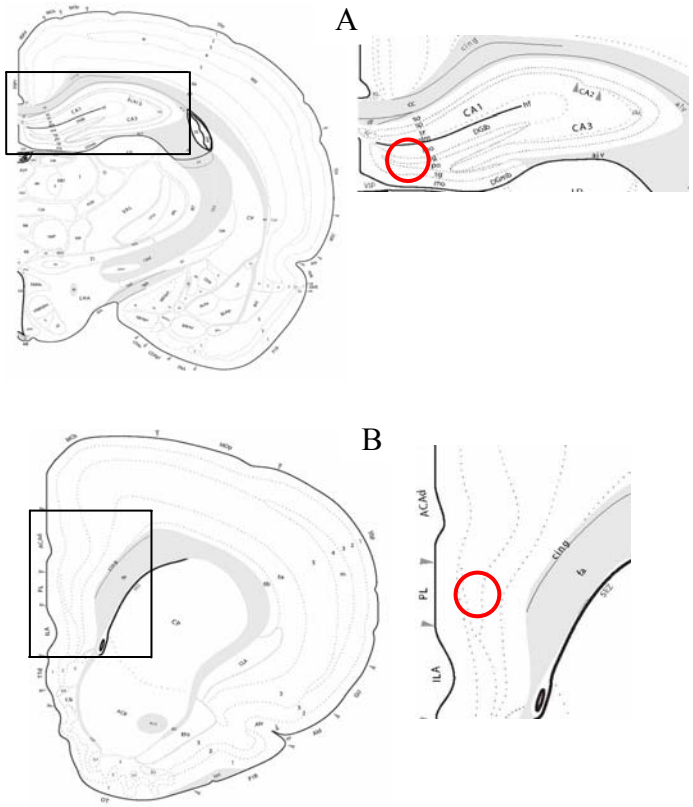


Figure 2. Atlas image represents the approximate brain level where dentate gyrus and prefrontal cortex was analyzed. A, dentate gyrus (dentate gyrus granule cell layer; DG-sg); B, prefrontal cortex (prelimbic; PL). The expression of CREB and pCREB was examined by Western blot or immunohitochemistry in the field circled by the red ring.

Immunohistochemistry

Following an overnight cryoprotection in a 30% sucrose solution, serial 30 μm coronal sections of the brain were made with a cryostat microtome and collected in 0.02 M potassium phosphate saline buffer (KPBS). CREB and pCREB immunostaining in DG and PFC was performed on free-floating sections. Sections were rinsed with 0.3% H_2O_2 for 10 min to reduce endogenous peroxidase activity, thoroughly washed with 0.1M PBS and incubated with the rabbit anti-CREB antibody (1:300, Cell Signaling) or anti-pCREB antibody (1:1000, Upstate) diluted in 0.1 M PBS with 0.1% Triton X-100 and 3% normal goat serum for 72 hours at 4˚C. After thorough washing, the sections were subsequently incubated for 2 hrs with biotinylated Goat-anti-Rabbit IgG (1:1000 in 0.1 M PBS with 0.1% Triton X-100 and 3% Normal Goat Serum) and avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit, Vector Laboratories). After thorough washing, the peroxidase reaction was developed with a DAB-nickel solution and 1% H_2O_2. Sections were washed for 15 min in buffer and mounted with a gelatine solution and air-dried, dehydrated in graded alcohol solutions and finally in Histoclear then coverslipped with DePeX mounting medium (BDH). To reduce staining artifacts or intensity differences the sections from all groups were processed simultaneously.

CREB or pCREB positive cells in the DG (dentate gyrus granule cell layer; DG-sg) (4 slices for each rat, bregma –2.45 to –2.85), and PFC (prelimbic; PL) (8 slices for each rat, bregma +3.20 to +2.15), were blindly quantified using a computerized imaging analysis system (Westenbroek et al, 2003a). The selected areas were digitized by using a Sony charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (Leica, Wetzlar, Germany) at ×100 magnification. Regions of interest were outlined with a light pen, measured and the CREB or pCREB positive nuclei were counted using a computer-based image analysis system LEICA (LEICA Imaging System Ltd., Cambridge, England). The resulted data was reported as number of positive cells/0.1mm^2. The DG and PFC was quantified bilaterally (no left-right asymmetry of CREB or pCREB ir was found).

Statistical analysis

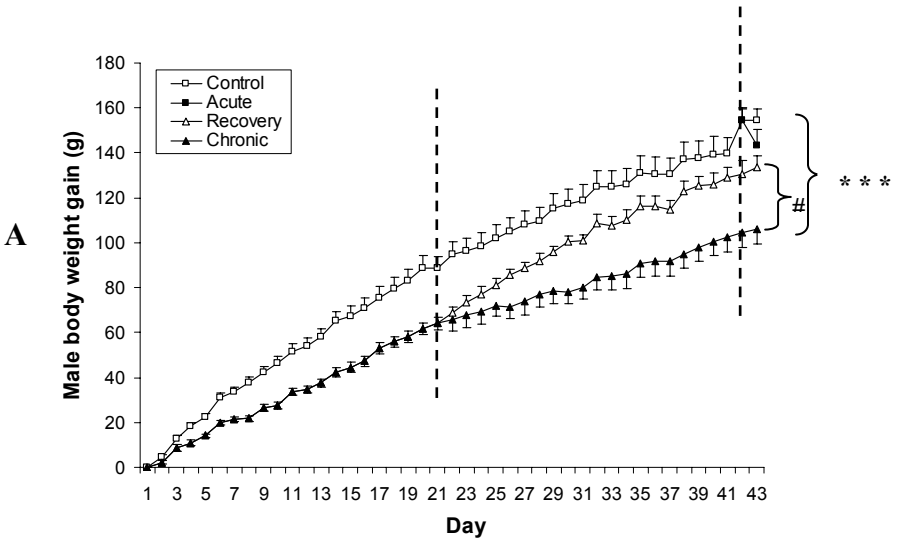
Data were expressed as means ± standard error (SEM) and analyzed with SPSS (version 12.0), $P<0.05$ was defined as the level of significance. Weight gain was analyzed separately for male rats and female rats with repeated measures ANOVA with days as within subject factors and treatment (control or stress) as between subject variables. Behavior was analyzed with a repeated measure ANOVA, with days as within subject variables and treatment (control or stress), gender (male or female) and housing

(individual or social) as between subject variables. Adrenal weight, plasma corticosterone levels, CREB and pCREB-ir were analyzed with an ANOVA with treatment (control or stress), gender (male or female) and housing (individual or social) as between subject variables.

Results

Body weight gain

All rats continued to grow throughout the experiment, and there was a significant effect of day on male and female body weight gain (resp. $F=1216.449$, $P<0.001$; $F=150.140$, $P<0.001$), and a significant gender difference with male rats growing faster than female rats ($F=490.839$, $P<0.001$). Body weight gain was significantly affected by chronic stress in male rats ($F=5.694$, $P=0.004$) but not in female rats, and the significant effect of stress on male body weight appeared after six days ($P=0.046$). In male rats there was a significant interaction effect between day and treatment ($F=10.096$, $P<0.001$). When stress was stopped, the recovery male rats restored their body weight, and the significance appeared from day 32 ($p=0.045$) to day 43 ($P=0.047$) compared to chronically stressed male rats, while there was no significance between control male rats and recovery male rats ($P=0.083$) at the end of the procedure. Social housing had no significant effect on the body weight of female rats (**Fig. 3**).



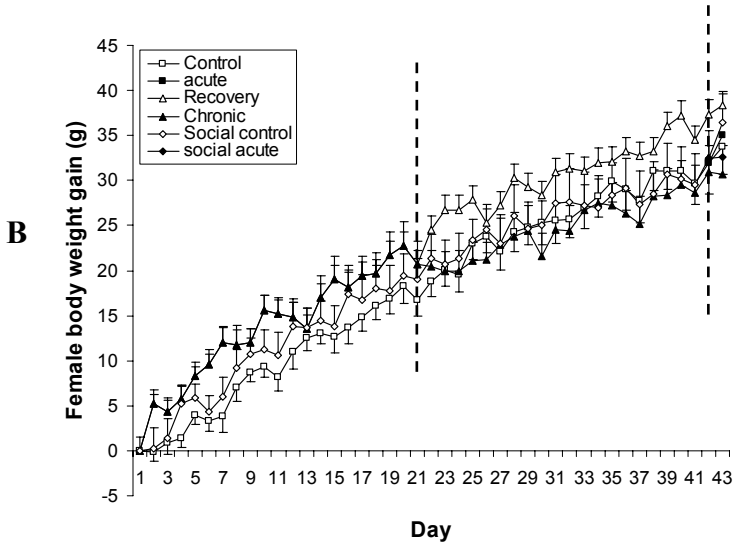


Figure 3. Effects of stress, recovery after long-term stress and social housing on body weight gain of male rats (A) and female (B) rats. Data were expressed as mean ± SEM, n=8. #P≤0.05, recovery group *vs* chronic stress group; ***P≤0.001 control group *vs* chronic stress group.

Adrenal and thymus weight

Gender difference was found in relative adrenal weight, the female adrenal was significantly larger than the male adrenal ($F=117.28$, $P<0.001$). Although chronic stress induced an increasing trend in male and female adrenal weight, no significance was found. Social housing also had no significant effect on female adrenal weight compared to individually-housed control female rats. However, there was a significant increase of relative adrenal weight in recovery female rats compared to control female rats ($P=0.029$) (**Fig. 4**). Changes in thymus weight were not significant after acute and chronic stress and recovery, also no gender difference in the relative thymus weight was found (data not shown).

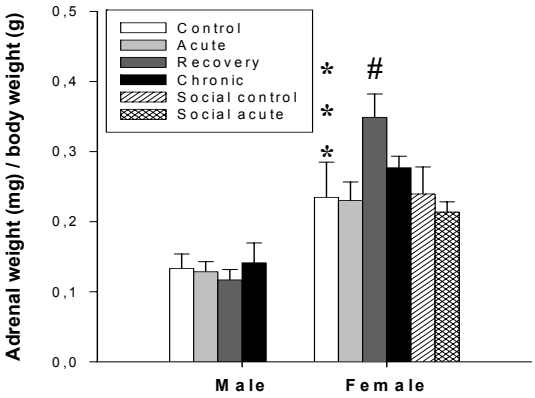


Figure 4. Effects of stress, recovery after long-term stress and social housing on adrenal weight measured on day 43 in male and female rats. Data were expressed as mean ± SEM, n=5. ***P≤0.001 *vs* control male group; #P<0.05 *vs* control female group.

Open field test

During the chronic stress procedure, there were no gender differences in the grooming frequency. The effect of day on grooming frequency was also not found. Exposure to stress for 3 weeks increased the grooming frequency compared to control group both in male rats ($F=3.726$, $P<0.05$) and female rats ($F=4.126$, $P<0.05$), which continued to increase after 3 more weeks from stress exposure in male rats ($F=5.263$, $P<0.01$) and female rats ($F=4.562$, $P<0.05$). As shown in **Fig. 5**, grooming behavior in male rats restored to the baseline levels after the stress was stopped for 3 weeks compared with chronic male rats ($P<0.01$). Although recovery female rats showed a slight decrease in grooming frequency, there was no significant reduction compared with chronically-stressed female rats and it was still increased compared to control female rats. Social housing had no significant effect on grooming behavior compared to individually-housed female rats (**Fig. 5**).

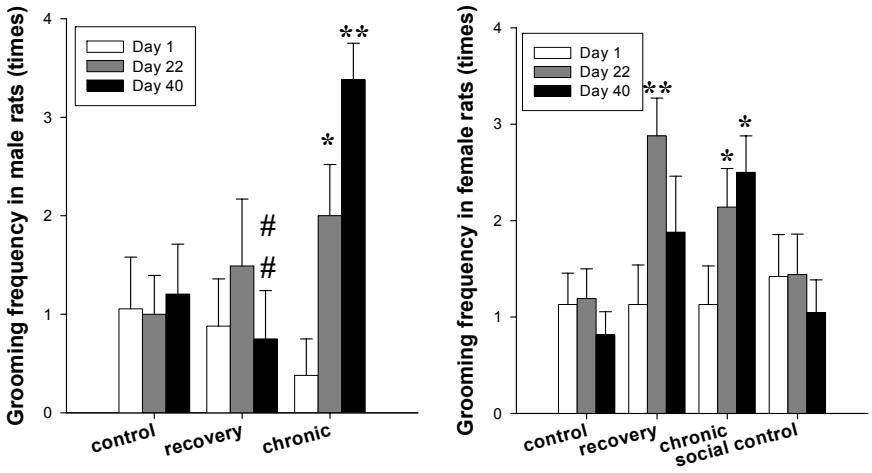


Figure 5. Effect of stress and recovery after long-term stress on grooming frequency in male and female rats. Chronically stressed male and female rats showed an increase in grooming frequency over time. Recovery after 3 weeks' stress restored grooming frequency in male rats while not in female rats. Data were expressed as mean ± SEM, n=8. *P≤0.05, **P≤0.001 within group, $^{\#\#}$P≤0.001 *vs* male chronic group.

Plasma corticosterone levels

0.5h after stress or exposure to stress box

Gender difference in plasma corticosterone levels was found both on day 20 and day 43 (resp. F=13.460, P<0.001; F=26.318, P<0.001), female rats showing significantly higher plasma corticosterone levels than male rats. On day 20 a significant increase in corticosterone level induced by chronic stress was observed both in male rats (F=26.826, P<0.001) and in female rats (F=4.629, P=0.009). On day 43, exposure to the conditional stimulus only significantly increased the corticosterone levels in male and female rats in acute stress group (resp. P=0.001; P=0.002), recovery group (resp. P=0.002; P<0.001) and the chronic stress group (resp. P=0.008; P=0.008) compared to control male and female rats respectively. There was no significant difference among the acute, recovery and chronic male and female groups on day 43. Social housing had no significant effect on plasma corticosterone level on day 20 and day 43 compared to the individually-housed female rats. However, exposure to the stress box induced no significant increase in the plasma corticosterone levels in social acute female rats compared to social control female rats (**Fig. 6**).

2h after exposure to stress box

Gender difference in plasma corticosterone levels was also found 2h after exposure to the stress box on day 43 (F=7.492, P=0.012). A significant decrease in plasma corticosterone levels was found in chronically stressed male rats (P=0.029) compared to control male rats. Furthermore, in acute group there was also a significant reduction in plasma corticosterone levels in individually-and socially- housed female rats (resp. P=0.028; P=0.035) (**Fig. 6**).

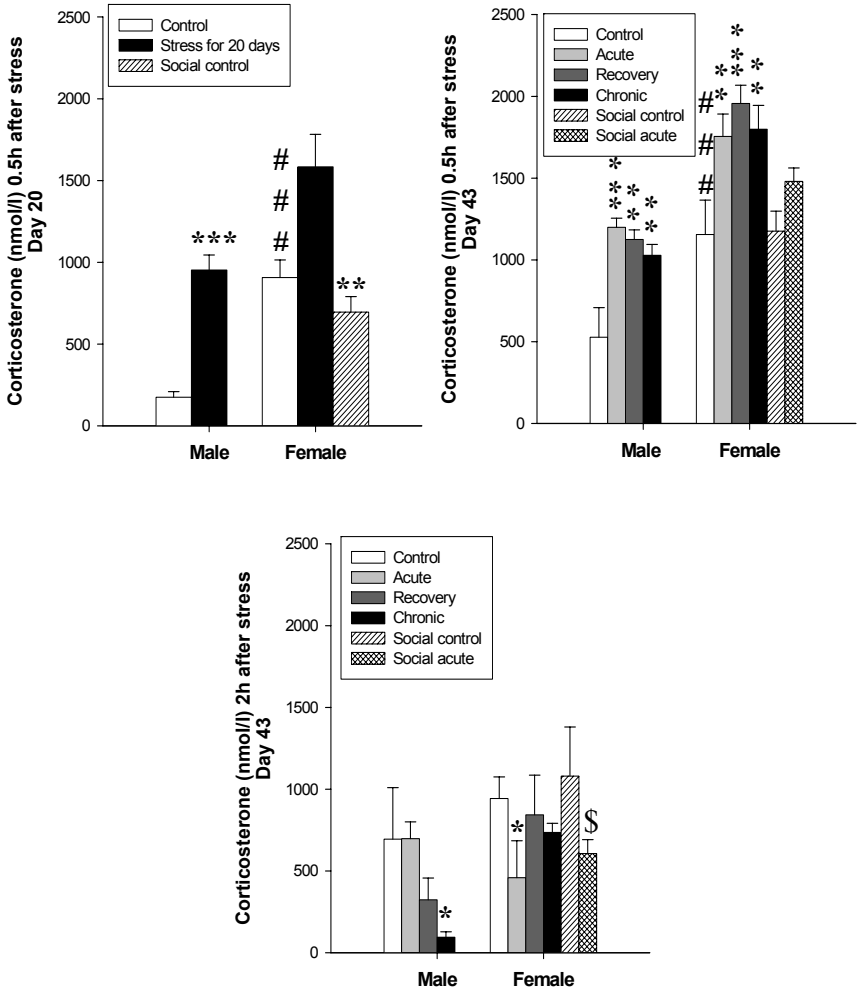


Figure 6. Effects of stress, recovery and social housing on plasma corticosterone levels measured 0.5 h after stress on day 22 (n=8), 0.5 h (n=5) and 2 h (n=3) after exposure to the footshock box with the light only on day 43. Data were expressed as

mean ± SEM. *P<0.05, **P<0.01 ***P<0.001 *vs* control group; # # #P<0.001 *vs* male control group; $P<0.05 *vs* social female control group.

CREB and pCREB expression in male and female PFC

Protein expression 0.5h after exposure to the stress box

Acute and chronic stress both had no significant effect on the expression of pCREB and CREB in male and female PFC, although chronic stress induced a decreasing trend in the expression of pCREB and CREB. Social housing also did not affect CREB and pCREB expression in female PFC (**Fig. 7**).

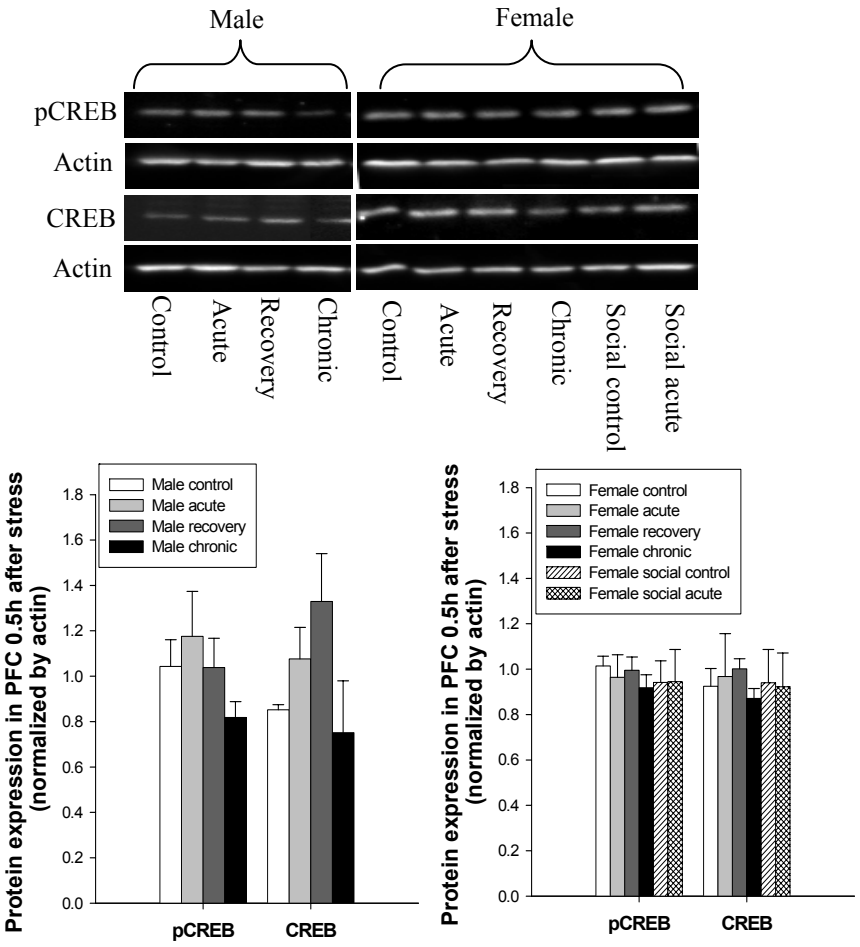
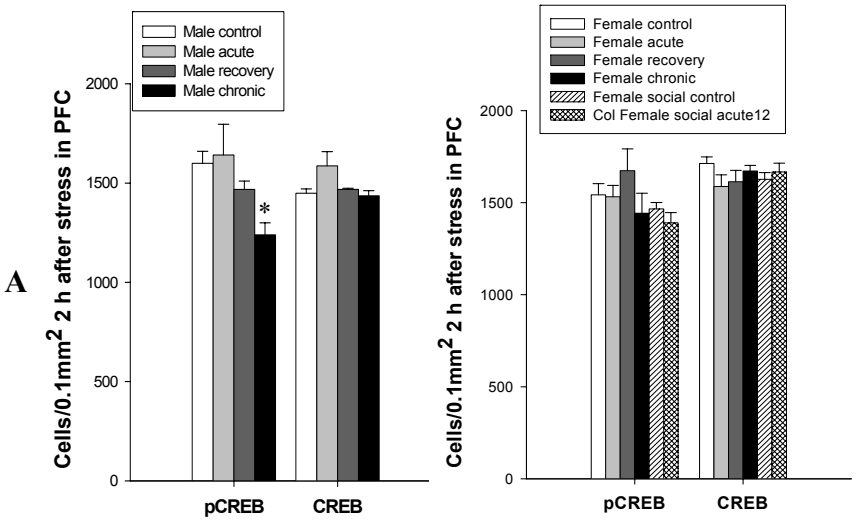


Figure 7. Expression of pCREB and CREB measured by Western blot (n=5) in prefrontal cortex (prelimbic) of male and female rats 0.5 h after exposure to the

footshock box with the light only on day 43. The intensity of Western bands was quantified with a densitometric scanner and each column represented the group means ± SEM of 4 determinations. Stress, recovery and social housing had no significant effect on the expression of pCREB and CREB in prelimbic 0.5 h after exposure to the footshock box.

CREB- and pCREB- positive cells number 2h after exposure to the stress box

There was a significant decrease in the number of cells expressing pCREB (P=0.023), while no significant change in the number of CREB-positive cells in PFC of chronically stressed male rats compared to control male rats. Chronic stress also induced morphological abnormalities and irregularities in male PFC which were reflected by the "air bubbles". After recovery there was no significant change in the number of cells expressing pCREB and CREB in male PFC. In female PFC no significant change in the number of cells expressing CREB and pCREB was found, also no "air bubbles" were examined (data not shown). Gender differences in the number of cells expressing CREB and pCREB in PFC were not found in this experiment (**Fig.8**).



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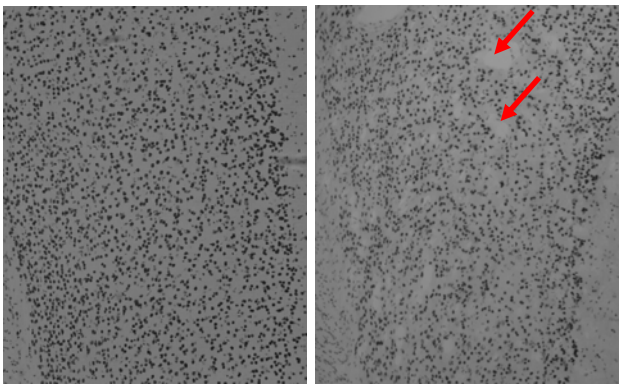


Male control (×100) Male chronic (×100)

Figure 8. Number of cells expressing pCREB and CREB in prefrontal cortex (prelimbic) measured by immunohistochemistry (n=3) in male and female rats 2 h after exposure to the footshock box with the light only on day 43. **A**, number of cells expressing pCREB and CREB 2h after exposure to the stress box on day 43 in male and female prelimbic, *P<0.05 *vs* control group. **B**, representative photomicrographs of pCREB-ir in the prelimbic of male rats exposed to chronic stress, air bubbles were clearly observed (red arrow).

CREB and pCREB expression in male and female DG

Protein expression 0.5h after exposure to the stress box

In male rats, acute and chronic stress slightly decreased the expression of pCREB in DG, but the effect was not significant. Stress also had no significant effect on the expression of CREB in male DG. In female DG stress and social housing had no significant effect on the expression of CREB and pCREB (**Fig. 9**).

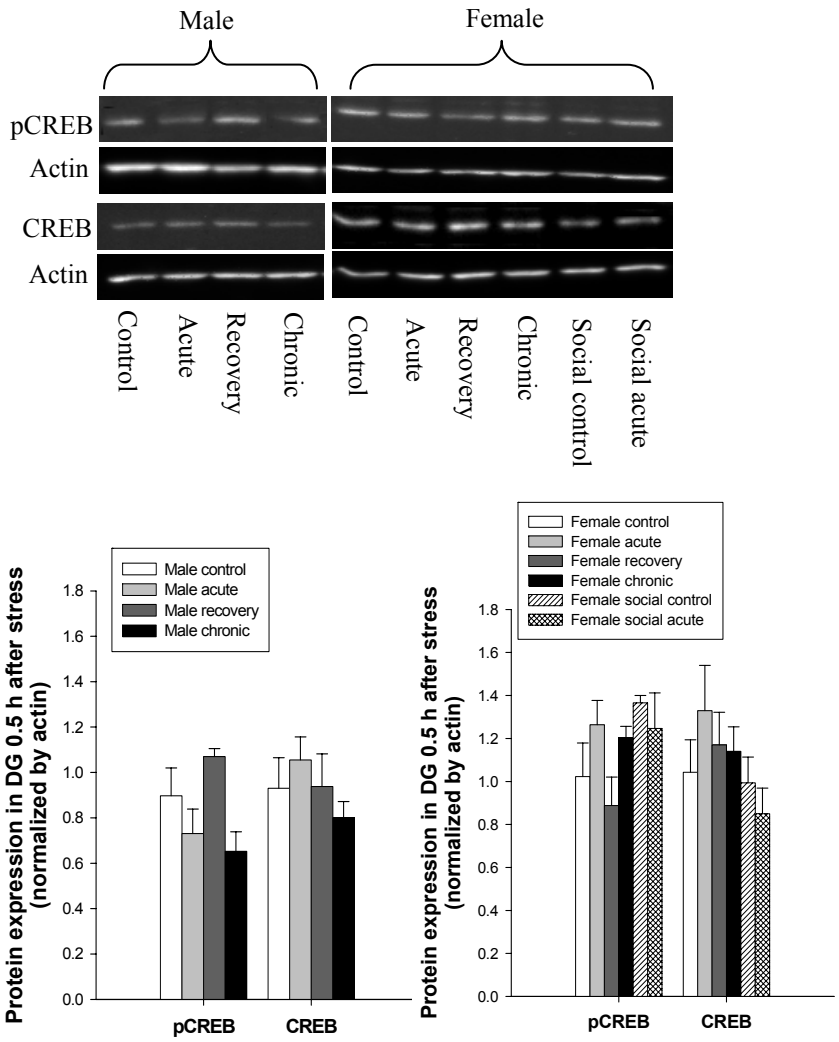
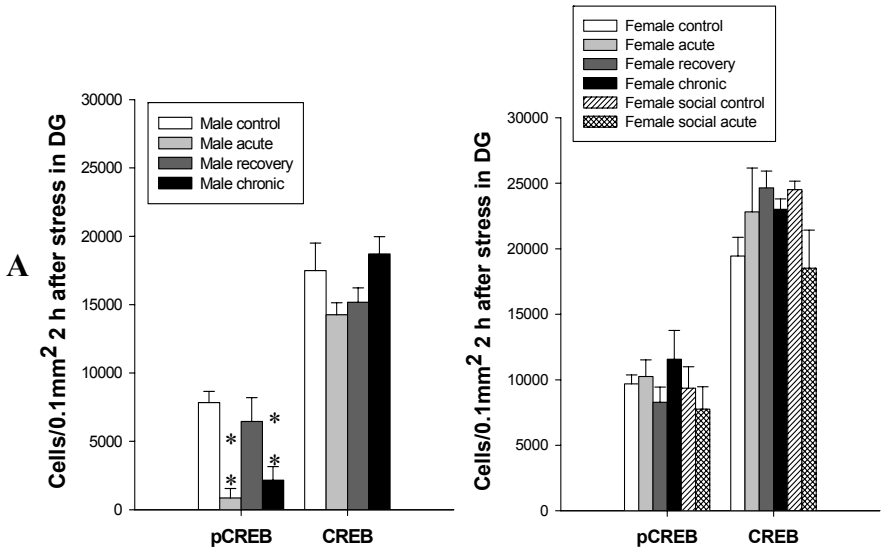


Figure 9. Expression of pCREB and CREB measured by Western blot (n=5) in dentate gyrus (dentate gyrus granule cell layer) of male and female rats 0.5 h after exposure to the footshock box with the light only on day 43. The intensity of Western bands was quantified with a densitometric scanner and each column represented the group means ± SEM of 4 determinations. Stress, recovery and social housing had no significant effect on the expression of pCREB and CREB in dentate gyrus granule cell layer 0.5 h after exposure to the footshock box.

CREB- and pCREB- positive cells number 2h after exposure to the stress box

In male rats, a significant effect of stress on the number of cells expressing pCREB was found in DG (F=8.673, P=0.007). The number of pCREB-positive cells was significantly decreased in acute (P=0.002) and chronically stressed male rats (P=0.008). Morphological abnormalities and irregularities were demonstrated by many "air bubbles" especially around the subgranule cell layer (SG) in male DG. However, in recovery male rats there was no significant change in the number of pCREB-positive cells compared to male control rats on day 43. Stress had no significant effect on the number of CREB-positive cells in male DG. In female DG there was no significant change in the number of CREB and pCREB-positive cells after acute and chronic stress, recovery and social housing compared to individually-housed control female rats (**Fig. 10**). Acute and chronic stress also didn't induce "air bubbles" in female DG (data not shown). Gender differences in the number of CREB- and pCREB-positive cells in DG were not found in this experiment.



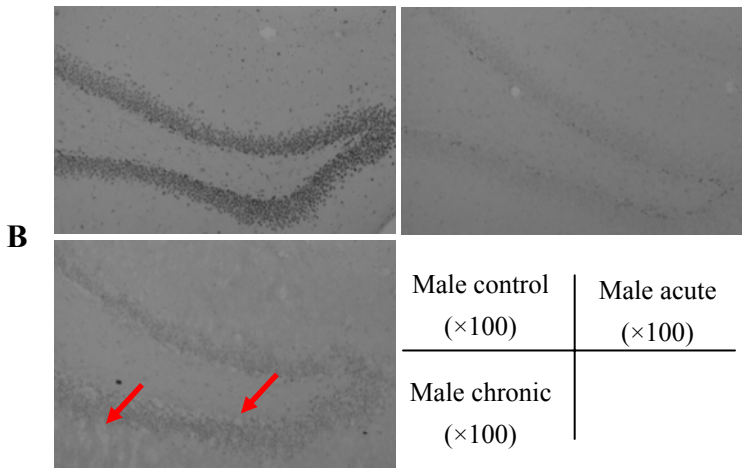


Figure 10. Number of cells expressing pCREB and CREB in the dentate gyrus (dentate gyrus granule cell layer) measured by immunohistochemistry (n=3) in male and female rats 2 h after exposure to the footshock box with the light only on day 43. **A**, number of pCREB- and CREB-positive cells 2h after exposure to the stress box on day 43 in male and female dentate gyrus granule cell layer, **P<0.01 *vs* male control group. **B**, representative photomicrographs of pCREB-ir in the dentate gyrus granule cell layer in male rats exposed to acute and chronic stress, air bubbles were clearly observed (red arrow).

Discussion

Despite the striking gender differences in the prevalence of depression, attempts to identify corresponding gender differences in stress response in animal models have met with limited success (Altemus, 2006). In the present study gender differences in response to stress and recovery after long-term stress were further investigated. Our results demonstrated that male and female rats coped with stress and recovery in a different way.

Effects of acute stress on individually-housed rats

Gender differences in the expression of CREB and pCREB in PFC and DG after acute stress were observed in this study. On the whole acute stress had no significant effect on the expression of CREB and pCREB in female PFC and DG. Acute stress

impacted the expression of pCREB time-dependently and caused a significant reduction in pCREB-immunoreactivity (ir) while not CREB-ir in male DG 2h after exposure to the stress box. These results suggest that DG and PFC responded to acute stress in a different way.

The gender difference in the expression of pCREB in DG after acute stress may be related to the changes in corticosterone level. Acute stress induced a significant increase in plasma corticosterone levels both in individually-housed male rats and female rats. However, 2h after stress individually-housed female rats while not male rats showed a significant reduction in the plasma corticosterone levels which may be due to increased negative feedback (Gunderson et al, 2003), suggesting that in female rats a protective mechanism may have become activated. Rises in corticosteroid levels after acute stress impair synaptic plasticity in the rat hippocampus when compared with the situation where levels are basal under rest (Alfarez et al, 2003), for hippocampus is a prime target for glucocorticoid action (De Kloet, 1995). The changes of pCREB subsequently may result in the alterations in neurotrophin expression, for CREB and pCREB play a central role in mediating neurotrophin responses in neurons (Finkbeiner et al, 1997; Finkbeiner, 2000). For examples, acute stress reduces neurotrophin expression in the hippocampus of male mice (Pizarro et al, 2004).

Activation of the infralimbic and prelimbic area of the PFC is necessary for stressor controllability (Amat et al, 2005). In a previous study acute stress was found to increase the expression of pCREB in PFC (Pardon et al, 2005), suggesting that protective effect of stressor controllability are mediated by the PFC. However, in the present study, although an increasing trend in the expression of pCREB in male PFC was observed, no significance was found. The differences from the previous study may result from the different breed of animal, stressor, methods used etc.

Effects of chronic stress on individually-housed rats

Stress generated an inhibitive effect on male weight gain, while no such effect was found in female rats. These results were in accordance with previous preclinical data showing that stress exposure did not affect body weight gain in female rats as much as it did in male rats (Duncko et al, 2001). Chronic stress induced behavioral changes such as increased grooming frequency both in male rats and female rats while no gender difference was found. The behavioral changes indicated a depressive state in male and female rats. Stress for 21 days induced adrenal hypertrophy both in male and female rats (Westenbroek et al, 2003a), while no significance was found after 43 days' stress in this study due to an unknown mechanism (Kioukia-Fougia et al, 2002; Kuipers et al, 2006).

As expected (De Kloet et al, 1998), acute and chronic stress induced classical features of prolonged elevations of circulating corticosterone levels from the adrenal gland 0.5h after stress or exposure to stress box. However, female rats demonstrated higher plasma corticosterone levels than male rats not only under normal condition but also during stress (Rivier, 1999; Tinnikov, 1999). Interestingly, 2h after exposure to the stress box plasma corticosterone levels were significantly decreased through an unknown mechanism (maybe negative feedback, breakdown etc) in chronically stressed male rats. In our experiment chronic stress significantly reduced pCREB expression in male DG but no obvious effect on female DG. While CREB expression remained unaffected by chronic stress both in male and female DG, suggesting that CREB activity in the hippocampus was insensitive to chronic stress (Miller et al, 2006). Consistent with our results, chronic mild stress (CMS) was also found to result in a significant decrease in the expression of pCREB in male DG, while the expression of total CREB was unaffected by CMS (Gronli et al, 2006). In view of the importance of CREB and pCREB in neuroplasticity, our results were also consistent with other studies showing that chronically restrained female rats did not exhibit the severity of apical dendritic atrophy that was seen in stressed males (Galea et al, 1997), and that stressed male vervet monkeys showed evidence of hippocampal pyramidal neuron loss while females did not (Uno et al, 1989).

Besides the hippocampus, the male PFC also showed neurochemical changes in response to stress. As revealed in the present experiment, pCREB expression was reduced in male PFC. pCREB expression was also reported to be reduced by chronic stress in PFC in male rats (Laifenfeld et al, 2005). Significantly reduced pCREB expression in both cortical regions suggested reduced synaptic plasticity in male rats. Recent animal experiments showed that chronic stress resulted in altered dendritic morphology, and reduced spine density in the PFC (Cook and Wellman, 2004; Radley et al, 2004; 2006). Through down-regulating pCREB expression, chronic stress may thus compromise PFC plasticity required for proper response and/or adaptation to (stressful) stimuli (Kuipers et al, 2003). “Air bubble” in male PFC and DG after chronic stress were observed, which may be due to the dysfunction of HPA axis and prolonged elevation of corticosterone, for treatment with the synthetic glucocorticoid dexamethasone results in neuronal loss and atrophy in the mPFC as well as in the granule cell layer of the dentate gyrus (Cerqueira et al, 2005). The “air bubble” observed in the present study suggested a rate of the neuronal loss or an appearance of pycnotic cells. Interestingly, we did not find obvious changes in morphology (air bubbles) and expression of pCREB and CREB in female PFC and DG. Based on the results from the expression of CREB and pCREB and structural changes (air bubble) in PFC and DG, it may be concluded that female PFC and

DG was resistant while male PFC and DG was susceptible to the aversive effects of chronic and acute stress.

Effects of recovery on individually-housed rats

Opposing effects of recovery were found in male and female rats justified by adrenal hypertrophy, behavior and neurochemical changes. The effects of stress on male rats were not permanent and once the stress was removed, different parameters applied in this study restored to the baseline level. McEwen also noted that the remodeling of the hippocampus in response to stress was largely reversible if chronic stress was terminated at the end of 3 weeks (McEwen, 2004). Surprisingly, recovery female rats showed even more adrenal hypertrophy and abnormal behavior, indicating female rats were still suffering of the consequences of stress and thus apparently exhibited a resistance to recovery. In a previous study of our research group, levels of accumulated ΔFosB in the mPFC of female rats had not returned to control levels following 21 days of recovery, which showed that the preceding stress period still had transcriptional consequences in this limbic area (Gerrits et al, 2006). All these data suggest that recovery for 21 days was not enough for female rats to recover.

In light of the effects of chronic stress and recovery, it seemed contradictory that on one hand female rats were resistant to these stress-induced neurochemical changes in DG and PFC, on the other hand female rats still showed stressed after recovery, suggesting that other factors are involved in females. Therefore, other proteins related to neuroplasticity or more brain regions besides DG and PFC in female brain should be investigated, especially brain regions containing estrogen receptors, such as amygdala and hypothalamus where ERα dominates and entorhinal cortex and thalamus where ERβ dominates, for compelling evidence now exists for estrogen's involvement in depression and schizophrenia (Ostlund et al, 2003). Estrogen and progesterone administration to ovariectomized rats resulted in elevated levels of pCREB and BDNF in hippocampus (Sharma et al, 2007; Franklin and Perrot-Sinal 2006). Furthermore, depression in females emerges especially at times of severe changes in plasma estrogen levels, like after pregnancy, prior to menses and during and shortly after menopause (Arpels, 1996; Halbreich and Kahn, 2001; Kessler, 2003), implying that gonadal hormones may provide protection in females during stress.

Effects of social housing on female rats

In this study social housing prevented significant increases of plasma corticosterone levels after acute stress, suggesting that social housing maintained the

HPA axis at lower activity. Social housing was also found to help female rats to cope with chronic stress (Westenbroek et al, 2003a; 2003b), and has positive effects on neuronal survival (Westenbroek et al, 2004). All these results indicated that social housing had positive effects on female rats, whereas contradictory effects of group housing on males were reported (Gariepy et al, 2002; Karolewicz and Paul, 2001; Chourbaji et al, 2005). Oxytocin could be a possible mediator of the positive effects of social housing since social contact in rats was showed to increase oxytocin release (Uvnas-Moberg, 1997). In humans social contact is also associated with increases in plasma oxytocin levels and has been found to reduce plasma cortisol levels (Turner et al, 1999; Carter, 1998).

Conclusion

Male rats and female rats responded to stress and recovery in a different way. In female rats more evidences are needed to argue logically on one hand female rats were more susceptible to depression, and on the other hand PFC and DG was relatively resistant to stress in female rats, at least with the repeated footshock stress used here. Therefore our focus should be expanded to more proteins besides CREB and pCREB and more brain regions besides DG and PFC in female rats to clarify the neurobiological mechanisms of stress coping in female rats.

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